PROCESS FOR CULTIVATING MITES, NUTRIENT PREPARATION FOR THIS PROCESS, AND PREPARATION OF ALLERGENIC EXTRACTS FROM THESE MITES

The present invention relates to a process for cultivating mites or acari with a view to producing allergenic extracts from mites, and nutrient formulations intended to be used in these processes.

Different species of mites are used to prepare allergenic extracts used in allergy formulations to act, for example, as *in vivo* or *in vitro* allergy tests, or in desensitising preparations given to the patients.

These mites include, in particular, the following species : *Dermatophagoides pteronyssinus, Dermatophagoides farinae, Blomia kulagini* or *tropicalis, Pyroglyphus africanus,* and *Euroglyphus maynei,* which are housemites feeding mainly on human skin scales or squamae.

Conventionally, these mites are produced using culture media containing suitably treated human scales autoclaved to inactivate viruses, bacteria and unconventional transmissible agents such as prions. The mites thus produced make it possible to obtain, by extraction, high quality allergenic extracts which are virtually free from allergens of other origins capable of producing cross-reactions which may falsify the tests or give rise to allergies.

Other methods of cultivating mites are known, using nutrient media based on proteins such as shrimp eggs or powdered pig's liver. These media produce satisfactory yields but contain non-acarian substances, particularly of animal origin, which may cause allergies and/or have an infectious potential.

Although the methods of inactivation used to treat

human skin scales intended for the cultivation of mites are extremely effective and contamination with conventional or unconventional infectious agents is highly improbable, it is nevertheless desirable to use nutrient media of non-human and non-animal origin and free from any elements which might be allergenic to cultivate the mites.

The present invention therefore sets out to provide a process for cultivating and producing mites, and especially mites of the species mentioned above, which minimises the risk of the presence of infectious agents of animal or human origin.

Another objective of the invention is to provide a process of this kind which results in a substantial yield of mites.

Yet another objective of the invention is to improve the antigenicity of the mites intended to be used or extracted to produce the final formulations.

Yet another objective of the invention is to enable a large proportion of the culture medium to be eliminated easily.

The invention relates to a process for cultivating and producing mites, and especially mites belonging to at least one of the following species : *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, *Blomia kulagini* or *tropicalis*, *Pyroglyphus africanus*, and *Euroglyphus maynei*, characterised in that the mites are cultivated on a medium free from human or animal elements or proteins and comprising, in effective amounts, a plurality of amino acids in particulate form with a particle size of less than 250 μm, or in lyophilised form.

The desired particle size of amino acids may be obtained by grinding the amino acids, individually or in admixture.

In equivalent manner, the amino acids may be obtained by dissolving the amino acids, and then, lyophilising them. In this case it is preferable for the lyophilisation to produce particles smaller than 250 μm.

Of course, in the medium according to the invention, only some of the acids may have been ground and/or lyophilised, especially as a function of their physical characteristics, for example their solubility, and, if need be, some acids may be added to the mixture just as they are.

The invention is based on the discovery that if commercially available amino acids are used just as they are (without grinding and/or without solubilisation-lyophilisation and/or without the addition of salts), the mites grow extremely poorly and the yields are very low. Surprisingly, the mixtures of amino acids having the features defined in the invention result in yields that are comparable or even superior to the conventional yields using human skin scales.

The mixtures of amino acids preferably comprise the majority or all of the amino acids which naturally constitute proteins. By the majority is meant at least 50%, for example 60 to 80%, of the twenty amino acids which naturally make up proteins or equivalent assimilable amino acids. However, it is also possible to add amino acids which do not constitute proteins, or to replace some of the amino acids with amino acids which do not constitute proteins.

In one particular embodiment, a mixture of amino acids resembling the composition of keratin or the stratum corneum may advantageously be used.

However, in alternative embodiments of the invention, it is also possible to use mixtures of amino acids mimicking the composition of shrimp eggs or soya.

Other formulations may move away from this distribution.

The respective proportions of the amino acids may resemble the quantitative proportions of the amino acids in substances such as keratin, or the stratum corneum, shrimp eggs or soya, but there is by no means any requirement that the proportional distribution should be identical, provided that the individual amino acids are present in sufficient amounts.

The nutrient medium which contains the mixture of amino acids in the process, according to the invention, may also contain other normal ingredients of nutrient media for mites, intended either to provide a nutritional supplement or to give the medium a texture suitable for the development and multiplication of the mites, as well as salts.

Thus, it is preferably to incorporate wheat germs and/or yeast, especially baker's yeast, and/or cyanocobalamine and/or d-biotin, in the culture medium. The medium may also contain other vitamins.

The wheat germs are preferably heated to eliminate any risk of an allergic reaction.

In the process according to the invention, the culture medium, containing the mixture of amino acids, is brought to a suitable level of humidity which is normal for the mites being cultivated, and is kept at the appropriate usual temperature. The cultivation times may be three months, for example, while conventional periods of 2 to 5 months are preferred.

The invention also relates to the culture media containing the mixtures of amino acids according to the invention.

The invention also relates to the processes for preparing extracts or formulations of allergens obtained from the mites cultivated by the process according to the

invention.

Further features and advantages of the invention will become apparent from reading the following description which is provided as a non-restrictive example.

Cultures were set up simultaneously on amino acid media according to Examples 1 and 3 and on human skin scales according to Example 2.

Example 1 :

This example describes a process of cultivation in a medium containing a commercial amino acid preparation.

A culture medium is prepared containing wheat germs, cyanocobalamine, baker's yeast and D-biotin.

The wheat germs are autoclaved at 121°C for 20 min. then ground and screened through a 250 µm mesh.

The cyanocobalamine is ground and screened through a 250 µm mesh.

The baker's yeast is heated to 122°C for 2 to 3 min., then to between 100 and 135°C for 12 sec. in a drum rotating at a speed of 5 rpm, then heated to 100°C for 15 min.

The commercial amino acid preparation was obtained from the company Frésénius-Kabi France S.A. It has the following composition, qs for 1 L of water PPI :

- L-alanine	3.8 g
- L-arginine	4.2 g
- L-aspartic acid	5.2 g
- L-cysteine hydrochloride monohydrate expressed as L-cysteine/L-cystine	1.7 g
- L-glutamic acid	11.5 g
- glycine	2.7 g
- L-histidine	3.1 g
- L-isoleucine	5.0 g

- L-leucine	6.7 g
- L-lysine hydrochloride, expressed as L-lysine	5.0 g
- L-methionine	2.4 g
- L-phenylalanine	7.0 g
- L-proline	10.3 g
- L-serine	9.6 g
- L-threonine	3.8 g
- L-tryptophane	1.3 g
- L-tyrosine	0.6 g
- L-valine	5.5 g
- calcium chloride dihydrate	0.44 g
- magnesium sulphate heptahydrate	0.493 g
- sodium hydroxide	2.6 g
- potassium hydroxide	0.70 g
- potassium chloride	0.078 g

The solution is lyophilised and the lyophilisate collected is screened through a vibrating screen with a pore size of 250 µm.

The medium itself is prepared as follows :

For 600 g of medium :

252 g of screened wheat germs, 252 g of screened yeasts, 90 g of the lyophilised and screened amino acid solution, 5.4 g of screened cyanocobalamine and 0.6 g of D-biotin are weighed, the whole is homogenised in a homogeniser and then passed through a screen with a mesh size of 400 µm. The medium is packaged in labelled stoppered bottles and can be kept in a cold room at temperatures between +2°C and +8°C.

The culture itself is carried out as follows :

The prepared media are seeded with a sample of conventional seed cultures of *Dermatophagoides pteronisynus*. Cultivation takes place in bottles at a temperature of 25°C, at a humidity level of 75%. The samples are taken at different times. The media harvested are

then lyophilised and extracted at 5% in a 4 g/l ammonium bicarbonate solution for 24 hours at +4°C, then centrifuged at 3000 rpm for 15 minutes at +4°C. The supernatant is harvested and then filtered on a Millex HV filter with a pore size of 0.45 µm (Millipore).

In the extracts obtained the total allergenic activity is titrated (by Rast inhibition), the proteins are titrated (by the Lowry technique and/or the Bradford technique), and the major allergens Der p 1 and Der p 2 are titrated (using conventional titration kits).

The results of the Rast inhibition are expressed as RI/ml (RI : reactivity index), the extracts being titrated in comparison with a reference extract the activity of which is 100 RI/ml.

Example 2 :

The medium of Example 2 is identical to that of Example 1 except that the lyophilised commercial amino acid solution is replaced by a preparation of human skin scales.

These scales are autoclaved in bags at 134°C for 18 min. The scales are then placed in stainless steel dishes and put into a drier at 37°C for 1 to 2 weeks. The scales are then ground up and screened through meshes of 500 and 250 µm.

The powdered scales obtained are then treated with acetone and left to decant for 24 hours. The supernatant is eliminated. Then a final washing operation is carried out using acetone. The residue is collected and spread in thin layers over dishes and covered with a perforated sheet of aluminium. This substance is dried under a cover and finally screened through a vibrating screen with a mesh size of 250 µm.

Instead of the 90 g of amino acid mixture, the

culture medium in this example comprises 90 g of the human skin scale preparation mentioned above.

Example 3 :

The culture medium contains wheat germs, cyanocobalamine and yeast as in Examples 1 and 2.

A mixture of amino acids is prepared as follows :

10 litres of sterile distilled water are poured into a container and the following amino acids are dissolved therein :

- L-alanine	17.2 g
- L-arginine	26.4 g
- L-cysteine hydrochloride monohydrate	4.4 g
- glycine	49.6 g
- L-histidine	5.2 g
- L-isoleucine	13.2 g
- L-lysine hydrochloride	20.0 g
- L-methionine	8.0 g
- L-proline	8.8 g
- L-serine	44.0 g
- L-threonine	13.6 g
- L-valine	13.6 g
- L-tryptophane	1.3 g
- L-phenylalanine	20.8 g
- L-leucine	34.8 g
- L-glutamic acid	64.4 g
- L-aspartic acid	9.7 g

The solution is then lyophilised and the lyophilisate collected is screened through a vibrating screen with a pore size of 250 µm.

6.5 g of aspartic acid and 4.0 g of tyrosine are ground up separately.

To prepare the medium, 79.5 g of the abovementioned

lyophilised mixture, 6.5 g of ground aspartic acid and 4.0 g of ground tyrosine are added to the 252 g of wheat germs, 252 g of yeast, 5.4 g of cyanocobalamine and 0.6 g of D-biotin.

The mixture is homogenised and then screened through a 400 µm mesh. After seeding, cultivation is carried out as in Examples 1 and 2.

The comparative results of Examples 1 to 3 are shown in Table 1 for a first cultivation cycle, after 3 months, and in Tables 2 and 3 for a second cultivation cycle (the mites cultivated on a given medium are re-seeded onto the same medium), after 2.5 months and 3 months, respectively :

TABLE 1

Total allergenic activity, protein levels, of Der p 1 and Der p 2 in 1/20th extracts of *Dermatophagoides pteronyssinus* cultivated on different media after three months' cultivation.

Medium containing	Amino acids (example 1)	Human squamae (example 2)	Amino acids (example 3)
Total allergenic activity (RI/ml)	263	284	573
Protein level (µg/ml ; Bradford technique)	537	544	217
Protein level (µg/ml ; Lowry technique)	5689	6446	5538
Der p 1 (µg/ml)	187.5	231.5	69.0
Der p 2 (µg/ml)	2.0	2.5	10.0

Table 2

Total allergenic activity, protein levels, of Der p 1 and Der p 2 in 1/20th extracts of *Dermatophagoides pteronyssinus* cultivated for a second cycle on different media after two and a half months' cultivation.

Medium containing	Amino acids (example 1)	Human squamae (example 2)	Amino acids (example 3)
Total allergenic activity (RI/ml)	617	713	195
Protein level (µg/ml; Bradford technique)	328	371	75
Der p 1 (µg/ml)	83.0	138.0	8.5
Der p 2 (µg/ml)	19.5	19.5	3.0

Table 3

Total allergenic activity, protein levels, of Der p 1 and Der p 2 in 1/20th extracts of *Dermatophagoides pteronyssinus* cultivated for a second cycle on different media after three months' cultivation.

Medium containing	Amino acids (example 1)	Human squamae (example 2)	Amino acids (example 3)
Total allergenic activity (RI/ml)	631	486	192
Protein level (µg/ml; Bradford technique)	363	411	110
Der p 1 (µg/ml)	206.0	267.5	23.0
Der p 2 (µg/ml)	14.0	4.5	5.0

It will be seen that, although cultivation on amino acids according to Example 3 gives good results during a first cultivation cycle, it gives poor results in a second cultivation cycle. This Example is therefore not

suitable for routine cultivation of mites.

By contrast, cultivation on amino acids according to Example 1 seems to be highly suitable, not only because the results are satisfactory after two cultivation cycles, but because they are very similar to the results obtained with human squamae, which constitute the natural food of the mites under discussion here. The relative weakness of the results obtained in the first test can certainly be put down to an excessively long cultivation period (harvesting at two and a half months would certainly have given better results).